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Amyloidosis and the Serum Amyloid A Protein Response to Muramyl Dipeptide Analogs and Different Mycobacterial Species

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Serum amyloid A protein (SAA) elevation accompanies induction of secondary amyloidosis in mice given *Mycobacterium butyricum* in Freund adjuvant. The synthesis of SAA by cultured hepatocytes is induced by a macrophage-derived mediator, which has been identified as interleukin 1. In these studies, SAA synthesis has been used as an index of macrophage activation to examine the in vivo response of mice to challenge with seven different mycobacteria and with synthetic analogs of the immunoadjuvant *N*-acetylmuramyl-L-alanyl-D-isoglutamine [MDP(L-D)]. SAA synthesis was stimulated by administration (by the intraperitoneal route) of the mycobacteria dissolved in saline, with *Mycobacterium vaccae* being the most active and *Mycobacterium leprae* being the least stimulatory. MDP(L-D), which is the minimal structure (molecular weight, 492) able to substitute for mycobacteria in Freund adjuvant, stimulated SAA synthesis, whereas the MDP(D-D) isomer was inactive. The butyl ester of MDP, which induces no detectable pyrogenicity but retains adjuvanticity, required a 100-fold greater dosage than MDP(L-D) in stimulating SAA synthesis. Amyloidosis was detected histologically only when active SAA inducers MDP(L-D), *M. vaccae*, and *M. butyricum*, were administered in incomplete Freund adjuvant, with amyloid-enhancing factor. These studies demonstrated that SAA elevation was a sensitive in vivo marker of the capacity of antigens to stimulate macrophages to produce interleukin 1. A point of considerable relevance to the human use of MDP was the observation that repeated injections of the adjuvant MDP in saline did not induce secondary amyloidosis.

Secondary amyloidosis is a well-recognized complication of many chronic inflammatory and granulomatous diseases, including tuberculosis and lepromatous leprosy (22, 23). The major constituent of the fibrils in secondary amyloidosis is the amyloid A protein (AA) (5). This nonimmunoglobulin fibril protein is deposited in the tissues and often remains unsuspected until the accumulation of amyloid in some appropriate site becomes sufficiently massive to compromise function and produce overt disease. The mechanism by which the AA protein accumulates in the tissue is not yet understood. The present studies were in part designed to investigate the role of mycobacterial products in the pathogenesis of secondary amyloidosis in mycobacterial diseases.

The immune deficiency found in patients with lepromatous leprosy (15, 29) has been ascribed to many factors including genetic unresponsiveness, suppressor T cells (27, 30), suppressor macrophages (7), defective chemotaxis (47), and various serum factors (31) which suppress proliferation of T cells in vitro. One of the serum factors which is present in high concentrations in patients with lepromatous leprosy is the serum AA protein (SAA) (18, 22–24). We sought to test the hypothesis that *Mycobacterium leprae* antigens induce elevated concentrations of SAA, which has been shown to cause suppression of T cell mediated immune responses in vitro (6).

SAA is the precursor of the amyloid fibril protein AA. It is synthesized as an acute-phase protein in the liver (25, 28, 35) after many different acute inflammatory stimuli. Evidence has also been presented for some SAA synthesis by neutrophils (33). SAA circulates in the serum in association with high-density lipoprotein (3, 4) and may be involved with endotoxin clearance,

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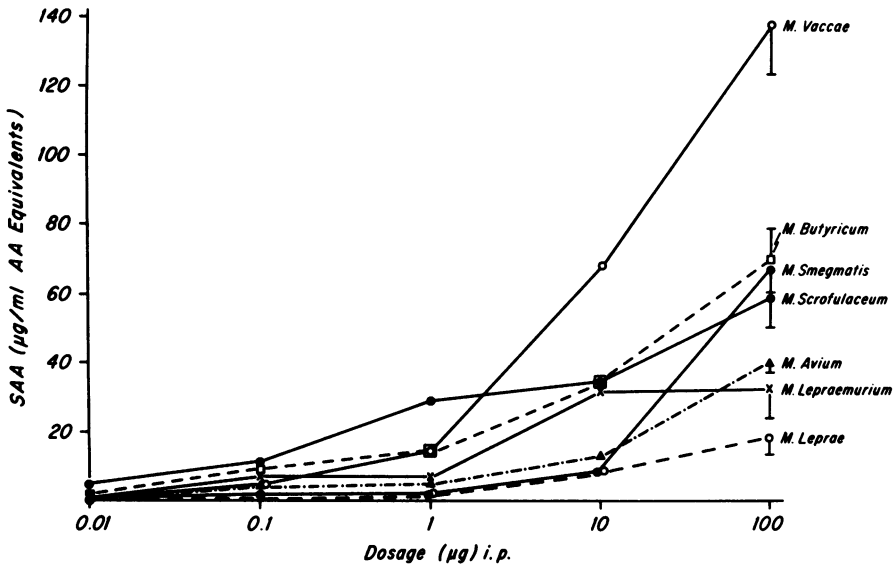

FIG. 1. SAA response of CBA/J mice injected i.p. with 0.2-ml volumes of different dosages of sonicated mycobacteria in saline. Animals were bled at 18 h from the retroorbital sinus, and serum was assayed for SAA. Data are given ± standard errors of the mean for three mice.

since it has been shown to bind to the same high-density lipoprotein molecules which contain endotoxin (lipopolysaccharide) (45).

SAA synthesis by hepatocytes is induced by a soluble factor, known as SAA-stimulating factor (SAA-SF) (25, 35) or SAA inducer (39), which is produced by macrophages. SAA-SF has recently been shown to be inseparable from the 15,000-dalton molecule which causes fever (leukocytic pyrogen) and activates lymphocytes (lymphocyte activation factor) (24, 42). This factor is also called interleukin 1 (IL1). Recent studies have identified a similar factor derived from a cultured keratinocyte tumor line (41); the in vivo significance of this source of SAA-SF–IL1 has yet to be shown. The clinical characteristics of diseases which lead to secondary amyloidosis can be accounted for on the basis of macrophage activation and IL1 activity, i.e., chronic diseases in which recurrent acute exacerbation is associated with fever, neutrophil leukocytosis, elevated concentrations of SAA, and hyperglobulinemia (23). It would be reasonable to predict, in lepromatous leprosy patients, for example, that a product of the abundant mycobacterial population (up to 10^{12} in a lepromatous leprosy patient) stimulates SAA synthesis and hence induces secondary amyloidosis.

In the present experiments, mycobacteria were administered to mice, and the acute phase protein response was monitored as a measure of the entire biological pathway from macrophage activation to production of SAA-SF to hepatic synthesis of acute-phase SAA. Injection of mice with complete Freund adjuvant is a standard amyloid induction regimen, and the active adjuvant moiety within complete Freund adjuvant is *Mycobacterium butyricum* (37). However, the minimal structure in mycobacterial cell walls which has been shown to give adjuvant activity is *N*-acetylmuramylalanyl isoglutamine (muramyl dipeptide [MDP]) (19). This 492.5-dalton adjuvant has now been synthesized, and various analogs were available for this study. We have shown that only those fractions known to stimulate macrophages in vitro (8, 9, 12, 14, 20, 21, 32, 43, 44, 46, 48) will induce an SAA response in vivo. The ability of different mycobacteria and MDP analogs to induce amyloidosis has also been assessed in a model of accelerated amyloidogenesis with amyloid-enhancing factor (AEF) (17). AEF has been purified as a non-AA component from the spleens of mice with secondary amyloidosis and has been used to induce amyloidosis within 5 to 7 days, when given in association with an inflammatory stimulus. We found that mycobacterial preparations which cause SAA stimulation lead to more deposition of amyloid in the spleen, as detected by radioimmunoassay.

MATERIALS AND METHODS

Mice. Female CBA/J, C57BL/10ScN mice (6 to 8 weeks old) were obtained from Jackson Laboratories, Bar Harbor, Maine. C57BL/10ScCr mice were obtained via our own breeding colony at Tufts from Sprague-Dawley, Madison, Wis.

Mycobacterial derivatives. MDP analogs were ob-

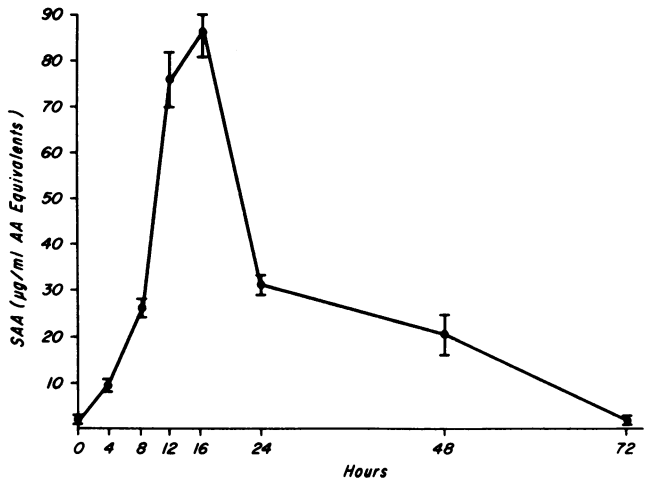

FIG. 2. Time response of SAA production after i.p. injection of 100 μg of MDP(L-D) in saline in CBA/J mice. Mice were bled at the times indicated, and serum was assayed for SAA. Data are given ± standard errors of the mean for three mice.

tained from S. A. Choay, Paris, France. *M. butyricum* was obtained from Sigma Chemical Co., St. Louis, Mo. Other mycobacteria were kindly provided by P. Draper, National Institute for Medical Research, Mill Hill, London (13).

SAA stimulation. Mycobacteria were suspended and sonicated in saline and injected intraperitoneally (i.p.) in a 0.2-ml volume. Animals were bled from the retroorbital plexus at the times indicated.

AEF production. AEF was extracted from the spleens of CBA mice which had amyloidosis. Spleens were homogenized in 4 M glycerol–10 mM Tris buffer (pH 7.6) shaken for 4 h at 4°C before centrifugation at 250,000 × *g* for 2 h at 4°C. The supernatant was dialyzed against phosphate-buffered saline (pH 7.6) for 24 h at 4°C, and the white precipitate which formed in the dialysis tubing was termed "crude AEF" (1, 17). This material was then concentrated and subjected to gel filtration on Sephadex G100 in 1 M glycerol–10 mM Tris buffer (pH 7.6) at room temperature. The void volume peak was pooled, dialyzed, concentrated, and used as semipurified AEF. This material was administered intravenously (i.v.) on the first day of amyloid induction at a dose of 20 μg i.v. in saline.

Amyloid induction. Three different sets of experiments were performed to examine the effects of mycobacteria and MDP analogs, with and without AEF in causing amyloid deposition. In one series of experiments, preparations were given by i.p. injection in saline for 17 days with and without prior i.v. AEF administration. In the second series of experiments, an 8-day induction period was used. After a single dose of AEF i.v., the mycobacterial preparations were given on 2 consecutive days i.p. at the start of the experiment. In the third regimen, the preparations were administered i.p. as an emulsion in Freund incomplete adjuvant on the first day of the experiment when AEF was given i.v.

Radioimmunoassay for AA. A solid-phase radioimmunoassay was used to detect AA cross-reactivity in the serum and splenic hemogenates (36, 38). Unknown samples were diluted in 500 μl of 10% (vol/vol) formic acid and incubated at 56°C for 24 h before the assay. Such treatment has been shown previously to expose AA cross-reactive determinants in tissue and serum samples. The assay was performed in plastic microtitration wells which had been coated with rabbit antibody affinity purified to mouse AA protein. Tween 20 (1%) was used to fill the unoccupied sites on the plastic, and the assay was performed in triplicate for each sample, with a standard curve for each plate. Portions of the formic acid-treated samples were lyophilized, dissolved in phosphate-buffered saline–Tween (pH 7.8) and applied to the wells in 20 μl, to which was added 5 ng of [^{125}I]AA. After incubation for 18 h at 4°C, the unabsorbed radioactivity was aspirated, the plates were washed, and the contents of individual wells were cut up into vials for gamma scintillation counting. A standard inhibition curve was obtained with each plate, and SAA values expressed as nanograms of AA equivalents per milliliter of serum or per total spleen wet weights.

RESULTS

SAA response to different mycobacteria. The SAA response to a 5 log dose range of each of the mycobacterial preparations administered by intraperitoneal injection is shown in Fig. 1. It is of note that *Mycobacterium vaccae* was significantly more stimulatory than the other preparations and that *M. leprae* caused the least stimulation of SAA production at 18 h. These differences could not be accounted for on the basis of endotoxin contamination, since *M. vaccae* caused an equivalent SAA stimulation in endotoxin nonresponder C57BL/10ScCR mice (27).

SAA time response of CBA/J mice after administration of MDP(L-D) (100 μg i.p.). *N*-Acetylmuramyl-L-alanyl-D-isoglutamine [MDP(L-D),

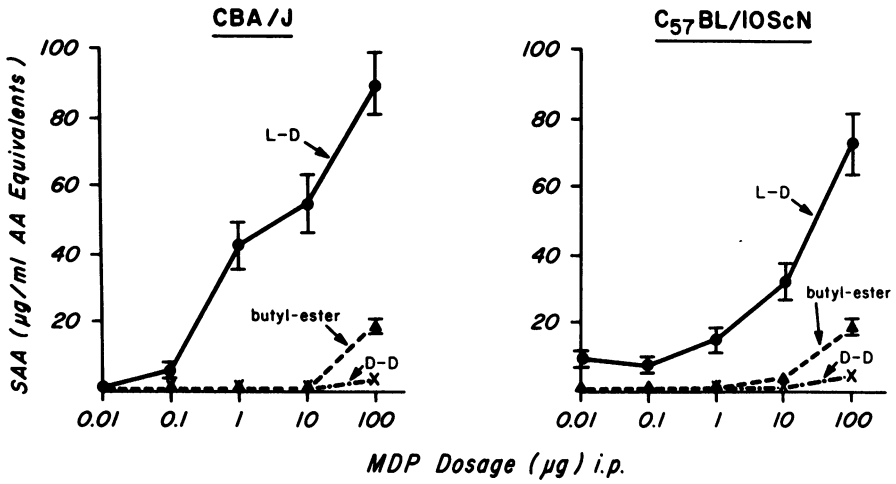

FIG. 3. SAA response of CBA/J and C57BL/10ScN mice after i.p. injection with 0.2-ml volumes of different dosages of MDP analogs. Mice were bled at 18 h from the retroorbital sinus, and serum was assayed for SAA. Data are given ± standard errors of the mean for three mice.

which is an active adjuvant analog of MDP, induced an SAA response which peaked at 16 h after administration of MDP (Fig. 2). The SAA levels returned to normal by 72 h. In further experiments, animals were bled at 16 to 18 h after MDP administration.

SAA responses of CBA/J and C57BL/10ScN mice to MDP analogs. Since previous experiments had shown that CBA mice were more sensitive to the mitogenic effects of MDP than were C57BL mice (11), these strains were compared by injection with MDP analogs in a 5 log dosage range (Fig. 3). Mice were bled at 18 h. Both strains responded equivalently to MDP(L-D). Neither strain responded to the MDP(D-D) isomer. The *n*-butyl ester of MDP was at least 100 times less potent in stimulating SAA synthesis than was the MDP(L-D) isomer. In another experiment the SAA response of C57BL/10ScN mice to MDP(L-D) was compared with the response of the syngeneic strain C57BL/10ScCR, which has been shown to be nonresponsive to endotoxin (26). However, both strains responded similarly to MDP(L-D) in terms of acute-phase SAA production. At 18 h after the administration of 100 μg of MDP(L-D) i.p., the endotoxin responder strain C57BL/10ScN produced 63.8 ± 6.2 μg of SAA, and the nonresponder strain C57BL/10ScCR produced 87.9 ± 30 μg of SAA, showing that the stimulus for SAA synthesis could not be accounted for by endotoxin contaminating the MDP preparations.

Amyloid induction in CBA/J mice. Previous well-defined regimens have used a single injection of *M. butyricum*-enriched complete Freund adjuvant to induce amyloidosis in mice within 10 days (36). Table 1 shows the results in CBA/J mice of 17 daily injections of MDP analogs or *M. butyricum* at a daily dosage of 100 μg per mouse in 0.2 ml of saline, with or without AEF. None of the three MDP isomers induced amyloidosis by histological criteria. However, the MDP(L-D) isomer induced significantly more AA in the spleen (assessed by radioimmunoassay), but not as much as was induced by a similar injection regimen with *M. butyricum*. Splenic AA concentrations were higher in the mice given AEF. Saline controls produced equivalent splenic AA to the MDP(D-D) isomer-treated animals and the MDP butyl ester-treated animals.

The AEF used in these experiments was shown to be active in accelerating amyloid induction by giving it (100 μg i.v.) at the same time as a single subcutaneous administration of silver nitrate 2% (0.5 ml). Spleens from these mice taken at 7 days showed histologically positive amyloidosis and 109.8 μg of AA per spleen. The SAA concentrations in the serum were also elevated at 7 days. AEF alone did not cause SAA synthesis.

Amyloid induction with AEF and mycobacterial derivatives. An 8-day induction protocol was used to compare the amount of amyloid produced by different mycobacterial species with the amount produced by MDP analogs after AEF administration i.v. at the start of the experiment. In an initial study (data not shown) each of the mycobacteria was given in saline on 2 consecutive days at the start of the induction experiment. Both MDP and mycobacteria given alone induced relatively modest increases in serum and splenic AA. However, with the addition of AEF, there was a marked increase in the AA protein detected in the spleen of animals

TABLE 1. Amyloid induction in CBA/J mice after 17 daily injections of MDP analogs with or without AEF

Substance injected[a]	Spleen AA[b] (μg/spleen)		Serum AA[b] (μg/ml of serum) with AEF[c]
	No AEF	With AEF[c]	
MDP(L-D)	0.405 ± 0.01	1.07 ± 0.21	5.92 ± 0.6
MDP(D-D)	0.09 ± 0.01	0.06 ± 0.002	1.8 ± 0.02
MDP (butyl ester)	0.110 ± 0.03	0.20 ± 0.02	1.44 ± 0.03
M. butyricum	0.649 ± 0.02	4.58 ± 0.31	5.1 ± 0.1
Saline	0.063 ± 0.01	0.09 ± 0.01	1.24 ± 0.01
Silver nitrate (at 7 days after 1 injection)		109.8 ± 5.6	35.3 ± 3.9

[a] Each i.p. injection contained 100 μg in 0.2 ml of saline.
[b] Sera and spleens were obtained at 25 days and assayed for AA reactivity by radioimmunoassay. Results are expressed as the means ± standard errors of the mean for three mice.
[c] AEF (200 μg) was administered i.v. in 0.5 ml of saline on the first day.

given MDP(L-D), *Mycobacterium smegmatis*, and *M. vaccae*, but none of the animals had histological evidence of amyloid deposition. Splenic AA concentrations could not be accounted for by the SAA present in blood. AEF alone and saline with AEF did not induce any increase in SAA or AA in serum or spleen.

In a final series of experiments animals were injected with different mycobacteria at a 10-fold higher dosage (1 mg per animal) as an emulsion in incomplete Freund adjuvant. Table 2 demonstrates the results at 8 days. When mycobacteria were administered in incomplete Freund adjuvant without AEF, there was an increase at 8 days in AA detected in the spleen of animals given *M. vaccae*, *M. butyricum*, and MDP(L-D). The amount of AA detected was equivalent to that found after AEF and 17 daily injections (100 μg/day) of these antigens in saline (Table 1). It was significantly more than the few nanograms of AA detected at 8 days after AEF plus the derivatives in saline (100 μg per animal). Again the SAA response to *M. vaccae* was consistently greater than to the other derivatives.

When AEF was given i.v. at the same time as the mycobacterial preparations in incomplete Freund adjuvant were given i.p., dramatically different results were obtained. Not only were elevated AA concentrations found in serum and spleen, but also deposition of amyloid in the spleens was observed in the animals treated with *M. vaccae*, *M. butyricum*, and MDP(L-D) in IFA.

DISCUSSION

It has recently been shown that SAA is synthesized as an acute-phase protein by cultured hepatocytes when they are stimulated with the soluble macrophage factor SAA-SF (35) or with purified endogenous pyrogen (25). Thus, in vivo production of SAA is directly related to the level of macrophage stimulation in mice. Assuming a normally responsive liver in the mice injected with myobacteria or MDP analogs, we have taken the serum concentration of SAA as a sensitive marker of macrophage stimulation (IL1 production) by the different preparations. It is clear that the response of macrophages to minor isomeric changes in the different MDP analogs is highly specific. The isomeric change of MDP(L-D) to the MDP(D-D) isomer converts an active macrophage-stimulatory molecule into a non-stimulatory molecule.

Recently, considerable interest has centered around the butyl ester derivative of MDP, which appears to stimulate human monocytes to produce lymphocyte activation factor, but not leukocytic pyrogen (8). Thus, although the butyl ester is an active adjuvant, it is markedly less pyrogenic. We therefore tested the capacity of this butyl ester to stimulate SAA synthesis in vivo. We have documented that the butyl ester is slightly more stimulatory than the MDP(D-D) isomer, but is 100 times less active (on a weight-for-weight basis) than MDP(L-D) in causing SAA synthesis. It seems, therefore, that the difference in biological activity between these MDP analogs is that the MDP(L-D) isomer stimulated macrophages to produce the soluble factor SAA-SF (IL1), whereas the MDP(D-D) isomer does not. The butyl ester will stimulate some SAA synthesis if large enough amounts are administered. Since the butyl ester of MDP retains its active adjuvant properties and is not pyrogenic in animals, it may be a promising candidate as an adjuvant for human administration.

It was of considerable interest to determine whether these potentially beneficial adjuvants induced amyloidosis when given by repeated injections. A model of accelerated amyloidogenesis has been developed by Kisilevsky et al. (17), who isolated an active AEF fraction from the homogenates of amyloidotic spleens. We

TABLE 2. Amyloid induction at 8 days after administration of AEF and mycobacterial derivatives in incomplete Freund adjuvant[a]

Substance injected	AA cross-reactivity[b]					
	Antigen alone			Antigen and AEF		
	Spleen AA (μg/spleen)	Congo red	Serum AA (μg/ml)	Spleen AA (μg/spleen)	Congo red	Serum AA (μg/ml)
M. vaccae	4.2 ± 0.8	−	336.4 ± 70	72.8 ± 22	++	241.2 ± 52
M. butyricum	3.1 ± 1.4	−	2.1 ± 0.6	104.2 ± 40	++	106.2 ± 42
MDP (L-D)	1.5 ± 0.3	−	6.1 ± 2.6	30.7 ± 6	+	132.5 ± 31
M. leprae	0.3 ± 0.02	−	2.8 ± 1.3	2.0 ± 0.4	−	35.6 ± 11
IFA[c] alone	0.2 ± 0.02	−	2.3 ± 0.5	1.86 ± 0.8	−	9.8 ± 2.4

[a] Injections of MDP and mycobacterial products (1 mg) were administered i.p. in an emulsion with incomplete Freund adjuvant on the first day of the experiment. AEF (200 μg in 0.5 ml of saline) was given i.v. to half the mice on the first day.

[b] Sera and spleens were obtained at 8 days and assayed for AA reactivity by radioimmunoassay. Results are expressed as the means ± standard errors of the mean for three mice. Spleens were examined histologically for amyloid deposition by Congo red staining: ++, strongly positive; +, weakly positive; −, negative.

[c] IFA, Incomplete Freund adjuvant.

have adapted his method of isolation of AEF and administered this enhancing factor i.v. at the start of induction experiments. When MDP analogs were administered in saline with or without AEF, MDP(L-D) induced more amyloid AA protein deposition than the others tested. However, the amounts were very small and did not approach the quantities required for histological identification. Thus, if it is reasonable to extrapolate to humans, it appears that there should be little risk that single or repeated injections of MDP will induce amyloidosis.

The clinical association between chronic mycobacterial infections and amyloidosis has been recognized for over 100 years. In previous studies, it has been shown that lepromatous leprosy patients who carry an overwhelming load of *M. leprae* bacilli are prone to developing amyloidosis, particularly when they suffer from acute episodes of erythema nodosum leprosum reactions (23). These erythema nodosum leprosum reactions are thought to be caused by immune complexes composed of antibodies to *M. leprae* and soluble antigens released from dead or dying bacteria. During erythema nodosum leprosum reactions, fever and neutrophilia are accompanied by elevated SAA concentrations (22). It was therefore of particular interest to compare different mycobacterial species in terms of their ability to stimulate SAA synthesis in our experimental model. The results indicate that there is a broad range of SAA-stimulating activity of different mycobacteria, with *M. vaccae* being the most active. It is noteworthy that *M. leprae* was the least active in this macrophage-dependent pathway. Although adjuvant activity has been noted in *M. leprae* preparations, this activity is not necessarily due to MDP. The relative amount of MDP in the different mycobacterial species is unknown, but it would be interesting to compare their MDP content with their capacity to stimulate macrophages and augment immune responses. MDP appears to be a rather nonantigenic determinant since antibodies to MDP have not been detected in patients with leprosy and tuberculosis (2). Another possible explanation for the apparent differences in the response of CBA/J mice to mycobacterial species is that there is independent control at the genetic level for response to different mycobacteria. Skamene et al. (40) have provided convincing evidence for the regulation of natural resistance to *Mycobacterium bovis* BCG by a single dominant autosomal gene, closely linked to a chromosome 1 locus for innate resistance to other intracellular organisms, including *Salmonella typhimurium* and *Leishmania donovani*.

Patients with lepromatous leprosy have a defect in cell-mediated immunity to the *M. leprae* bacillus. The mechanism for this immune deficit is still unclear, but is probably multifactorial (7, 10, 15, 27, 29–31, 48). Evidence exists for T suppressor cells directed at *M. leprae*-responsive lymphocytes and serum factors, including acute-phase proteins which can non-specifically suppress immune responses. Convit et al. (10) have found that the defect in macrophage handling of intracellular *M. leprae* can be corrected via local activation of macrophages by other species of mycobacteria. This exciting finding offers direct evidence that immunization with a species of mycobacteria that can stimulate macrophages may provide effective bypass signals to reverse the normally suppressed cell-mediated immune pathway of lepromatous patients. The mechanisms of this reversal of suppression and induction of macrophage activity are still conjectural. Our experiments would not support the hypothesis that the defects in cell-mediated immunity were related to elevated concentrations

of SAA, directly induced by *M. leprae* antigens. It appears that *M. leprae* are not particularly stimulatory to macrophages compared with the other mycobacteria species tested. It is more likely that immune complexes present during erythema nodosum leprosum reactions are the active stimulants for SAA-SF (IL1) production by macrophages and thereby cause SAA synthesis.

Induction of amyloidosis with mycobacteria in the accelerated amyloid model used in the present studies showed that preparations which stimulated the most SAA synthesis at 18 h were the most active in causing AA deposition in the spleen at 8 days and 17 days. With sonicated mycobacteria and MDP analogs in saline, there was insufficient amyloid deposition to detect histologically. However, using the radioimmunoassay for AA, submicroscopic concentrations of splenic AA could be detected quantitatively. When mycobacteria or MDP analogs were given in incomplete Freund adjuvant, the level of AA in the spleens was enhanced, concurring with previous reports that MDP is biologically more active when administered as an emulsion in oil.

The mechanism by which AEF causes amyloidosis is still poorly understood, but it would fit current hypotheses to suggest that the inhibition of enzymes which catabolize SAA might be responsible. Others have described an amyloid-degrading factor in serum which cleaves amyloid A fibrils in vitro. Patients with AA amyloidosis have elevated serum concentrations of an inhibitor of amyloid-degrading factor. It is unclear whether this serum inhibitor shares identity with the tissue-derived AEF (16). Recent experiments have demonstrated that patients with secondary amyloidosis have a distinctive pattern of catabolism of SAA in vitro when their monocytes are incubated with exogenous SAA (19). Thus, amyloidosis appears to be a two-stage process: the first stage is induction of high concentrations of SAA by any stimuli which cause macrophages to produce IL1, and the second stage involves the inhibition of catabolic enzymes for the degradation of SAA and AA fibrils.

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